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# THE EFFECTS OF SELECTED CELL OSMOLYTES ON THE ACTIVITY OF LACTATE DEHYDROGENASE FROM THE EURYHALINE POLYCHAETE, *NEREIS SUCCINEA* [1]

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The most commonly studied intracellular osmolytes of animals are the major inorganic ions, namely potassium (K), and, occurring at lower concentrations, sodium (Na) and chloride (Cl). In muscle tissue, which in most animals is the preponderant cell type in terms of body volume, the intracellular concentrations of these ions, particularly K, are relatively constant despite wide variations in total cell osmolarity (Burton, 1968, 1973, Figure 1). In the euryhaline polychaete, *Nereis succinea*, for example, the total intracellular concentration of these three ions ranges from 315 mM in animals adapted to 100% sea water (SW) to 147 mM in animals adapted to 10% SW. Potassium ion concentrations over this salinity range are decreased only in response to incomplete cell volume regulation with osmotic dilution (Freel, Medler and Clark, 1973).

Since the plasma or hemolymph ion content of a wide range of animals varies from about 300 mOsmol for freshwater species to >1000 mOsmol for marine species (Prosser, 1973), the intracellular osmotic activity of species with high body fluid osmotic pressures must be provided by solutes other than inorganic ions. Low molecular weight nitrogenous solutes comprise a major fraction, up to 0.6 M, of the intracellular solutes of marine invertebrates (Shaw, 1958; Jeuniaux, Duchâteau-Bosson and Florkin, 1961; Robertson, 1961; Awapara, 1962; Clark, 1968a; Freel *et al.*, 1973). Aside from the phosphagens, which are relatively constant at all osmotic dilutions and comprise some 64–82 mM in *Carcinus* (Shaw, 1958) and 12–14 mM in *Nereis* (Freel *et al.*, 1973), the remaining nitrogenous solutes are mainly amino acids, taurine, betaine, trimethylamine oxide (TMAO) and, in vertebrates, also urea (Lutz and Robertson, 1971; Robertson, 1975, 1976). Among euryhaline species, the concentrations of these solutes are regulated during adaptation to reduced environmental salinities (Shaw, 1958; Jeuniaux *et al.*, 1961; Virkar, 1966; Clark, 1968b; Freel *et al.*, 1973; Schoffeniels, 1976). So far, however, no explanation has been proposed for the function of these organic nitrogenous osmolytes, the maintenance of which at such high intracellular levels, in preference to readily available inorganic ions, must represent a significant energy expenditure by the organism.

Neutral salts have long been known to differ in their effects on the conformation of a wide variety of macromolecules including globular proteins and enzymes, fibrous proteins such as collagen, model polypeptides, and nucleic acids. In the Hofmeister or lyotropic series, cations such as $NH_4^+$ and $(CH_3)_4N^+$, and anions such as $SO_4^=$

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and $CH_3COO^-$ favor the native, functional state; the environmentally common ions, such as $Na^+$, $K^+$ and $Cl^-$ are less favorable; and ions such as $Ca^{++}$, $Mg^{++}$, $I^-$, $ClO_4^-$ and $SCN^-$ are distinctly deleterious at comparable ionic strengths (von Hippel and Schleich, 1969). As shown in Figure 1, the functional groups on the nitrogenous solutes accumulated by marine invertebrates are comparable to $NH_4^+$ (amino acids, taurine); to $(CH_3)_4N^+$ (TMAO, betaine); to $SO_4^=$ (taurine) and to $CH_3COO^-$ (amino acids, betaine). One can therefore postulate that high intracellular concentrations of $K^+$, $Na^+$ and $Cl^-$ would be injurious to macromolecular function and that these organic solutes serve a protective, or at least substitutive role in animals with blood osmotic pressures significantly greater than 300 mOsmol.

Most studies of specific ion effects on macromolecular conformations have been concerned with gross conformational changes, such as helix-coil transitions, that are observed only at far greater temperatures and ionic strengths (von Hippel and Schleich, 1969) than those experienced by most osmoconforming euryhaline invertebrates. It is therefore of interest to know whether lower concentrations of lyotropically less favorable solutes can effect grossly undetectable, yet functionally significant conformational changes in biological macromolecules. Some evidence that this is indeed the case exists in the observation by Warren, Stowring and Morales (1966) and by Warren and Cheatum (1966) of significant inhibitory effects of relatively low (0.5 to 1.0 M) concentrations of biologically prevalent salts, such as NaCl and KCl, on the activities of a number of vertebrate enzymes. Such observations have been confirmed recently by studies of neutral salt effects on enzymes of varying origin (Borowitzka and Brown, 1974; Somero and Low, 1977; Somero, Neubauer and Low, 1977).

The experiments described here were undertaken to test the following hypotheses: first, that an intracellular macromolecular of a marine invertebrate is indeed susceptible to functional inhibition by concentrations of those neutral salts found in the animal's environment and its extracellular fluid; secondly, that lyotropically more favorable salts, such as ammonium sulfate, or nitrogen-containing organic solutes with comparable functional groups, such as amino acids or TMAO, are innocuous to macromolecular function; and thirdly, that this latter group of molecules can offset the effects of functionally deleterious solutes. Examination was made on the functional behavior, in the presence of various solutes, of a representa-

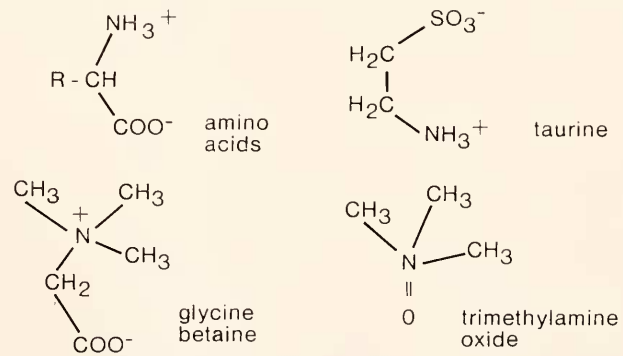


FIGURE 1. Formulas of intracellular nitrogenous solutes commonly found in marine invertebrates.

tive macromolecule, lactate dehydrogenase (LDH) (EC 1.1.1.27) obtained from the body wall tissues (mainly muscle) of a euryhaline polychaete, *Nereis succinea,* adapted to a wide range of salinities. This species is an osmoconformer at salinities greater than 30% SW and a partial regulator at lower salinities (Oglesby, 1965).

## Materials and Methods

### *Collection and acclimation of animals*

Specimens of *Nereis succinea* were collected from the Colorado Lagoon, Alamitos Bay, Long Beach, California between June, 1971 and January, 1973. They were maintained at 15 ± 2° C, without food, in artificial sea water, salinity 34.8‰, of the following composition: Na, 487.2 mM; K, 9.0 mM; Ca, 9.1 mM; Mg, 48.4 mM; Cl, 556.1 mM; $SO_4$, 25.5 mM; and $HCO_3$, 2.1 mM. Lower salinities were obtained by dilution with distilled water. Animals were adapted stepwise through 10% intervals of sea water dilution, remaining at least three days at each concentration and at least one week at the final acclimation salinity before being used. No animals were kept more than two months.

### *Enzyme preparation*

In general, large specimens were preferred for taking tissue samples, but fully mature females and heteronereids were excluded. Pooled tissues were taken from several animals adapted to each of the following concentrations of sea water: 100%, 75%, 50%, 35% and 20%. After removal of gut and parapodia, the body wall tissue was rinsed, blotted and weighed. From dry weight analyses on subsamples, appropriate amounts of 0.25 M sucrose were added to the weighed tissues to give a final dry weight of 21.3 μg per milliliter of homogenate prior to centrifugation. Following homogenization, supernatants were obtained by centrifugation at 22,000 × *g* for 30 minutes. All procedures were carried out at 4° C or less. Each supernatant was pipetted into several small vials and stored at −10° C until use. Although a single freeze-thaw cycle had no effect on LDH activity, repeated freezing and thawing resulted in significant loss of activity and was avoided.

Homogenates from animals collected at different times tended to vary in absolute activity. Although the cause of this variation is unknown, it did not appear to affect the conclusions reached in this study, since the relative effects of assay variables (pH, ionic strength, solute composition) on activity remained constant from batch to batch.

### *Enzyme assay*

The rate of pyruvate reduction by NADH was assayed by following the decrease with time in absorbance at 340 nm, using a Cary Model 15 recording spectrophotometer. In 1.04 ml of solution, the final composition was: 1.0 ml of $1.44 \times 10^{-4}$ M reduced coenzyme dissolved in a selected buffer with or without added osmolyte; 0.04 ml of varying concentrations of sodium pyruvate; and 2 μl of supernatant containing LDH. The reaction was initiated by the addition of pyruvate, following five minutes thermal equilibration during which any endogenous substrate was removed. The initial, linear (zero-order) change in absorbance was measured at

all substrate concentrations, and there was a linear increase in the reaction rate with an increase in enzyme concentration.

During preliminary studies, it was found that LDH in the absence of added osmolytes exhibits hyperbolic kinetics with increasing pyruvate concentration. Substrate saturation occurs at about $5 \times 10^{-3}$ M pyruvate and slight inhibition was observed at $5 \times 10^{-2}$ M. A concentration of $10^{-2}$ M pyruvate was therefore used to obtain maximum reaction rates. The optimal NADH concentration is $1.38 \times 10^{-4}$ M. All assays reported here were conducted at room temperature, and no thermal inactivation was observed up to 30° C.

Data are expressed either as change in optical density/minute per 2 $\mu$l of supernatant (the equivalent of 0.043 $\mu$g dry weight of tissue); or as rates relative to a control rate defined as the reaction velocity in the absence of added osmolytes. All comparative results were obtained at a single, constant temperature, using the same enzyme preparation.

Sodium pyruvate and NADH were obtained from Sigma Chemical Company. NADH was freshly prepared within four hours of use. All salts were reagent grade and nitrogenous solutes were A grade. TMAO was synthesized for us in the laboratory of Dr. Edward Grubbs, Chemistry Department, San Diego State University. Sorenson's buffers were prepared using sodium and potassium phosphates in a ratio of 1:3, to approximate intracellular ratios of these cations.

## Results

### *Effect of buffer composition and pH*

The optimum pH for LDH activity was determined in duplicate on homogenates from worms adapted to 100%, 75%, 50%, 35% and 20% SW. LDH supernatants obtained from *N. succinea* adapted to 100% sea water are designated as "100%" LDH; LDH from worms adapted to 20% sea water as "20%" LDH; and so on. Two types of buffer were utilized for determining optimum pH: Sorenson's phosphate buffer and tris-hydroxymethyl amino methane hydrochloride buffer (TRIS). Measurements were made over the pH range of 6.0 to 8.0, using zero order (saturation) kinetics.

The effects of pH, buffer composition and buffer strength on LDH activity from animals adapted to various salinities are shown in Figure 2. In both Sorenson's and TRIS buffers, there is no sharply defined pH optimum for any of the enzymes. The absolute amounts of enzyme are not identical for the two buffers because the homogenates were prepared from two different collections of worms. It is interesting that, in both cases, LDH activity per 0.043 $\mu$g dry weight of homogenate is considerably higher for osmoregulators (20% LDH) than for osmoconformers. This cannot be explained by the relatively small effect of amino acid loss during osmotic dilution (Freel *et al.*, 1973) on the dry weight composition, and suggests either an increased synthesis of the same enzyme or, perhaps, synthesis of a new isozyme by osmoconforming worms.

The effect of TRIS buffer at strengths ranging from 0.001 to 0.1 M on a single batch of "100%" LDH at pH 7.0 is compared with that of 0.03 M Sorenson's buffer and 0.1 M trimethylamine (TMA) and triethylamine (TEA) in Figure 2c. There is no difference between the three organic buffers. Increasing organic buffer

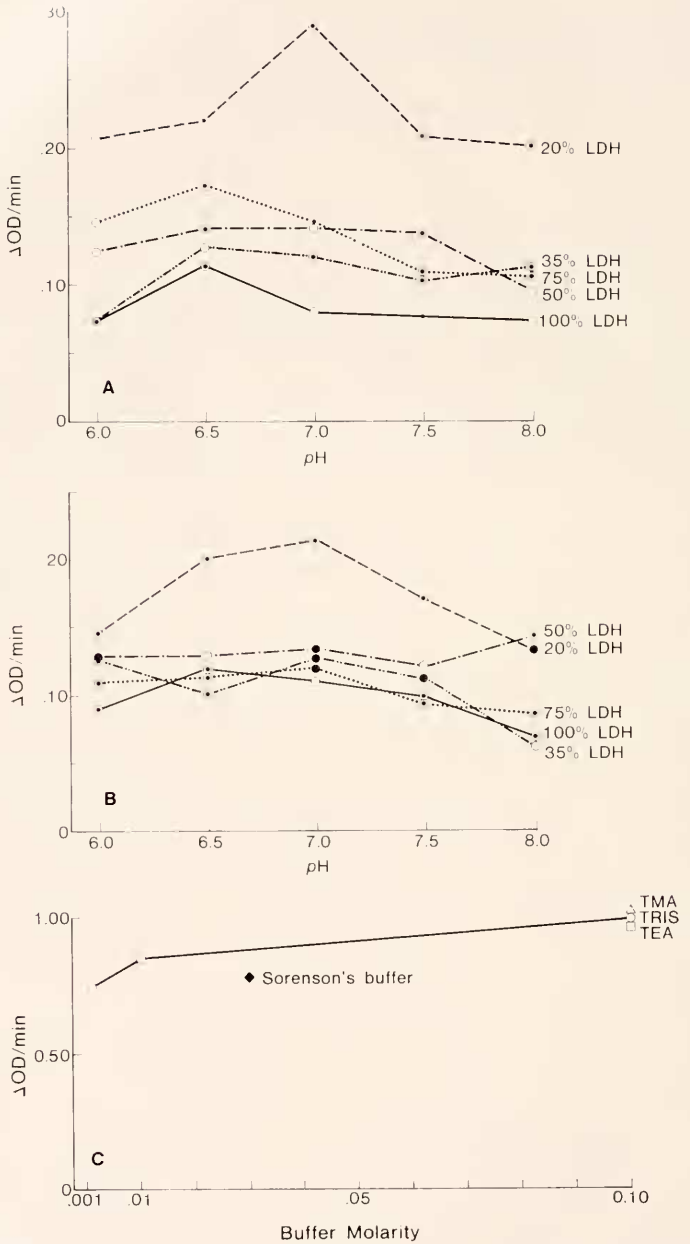


FIGURE 2. The effect of pH, buffer composition and buffer strength on LDH activity: effect of pH in (a) 0.03 M Sorenson's buffer and (b) 0.1 M TRIS buffer; effect of buffer strength (c) on "100%" LDH activity at pH 7.0. Symbols for replicate agreement, which apply to (a) and (b), are: circle with dot, two replicates differ by >20%; filled circle, two replicates differ by >10%; open circle, two replicates differ by <10%; and dot, single determination.

strength increases activity, and Sorenson's buffer appears to be slightly less favorable than TRIS.

All experiments on the effects of added osmolytes were carried out at a pH of 7.0, near the middle of the pH optimum for all homogenates, using organic buffer (TRIS, TMA or TEA) at 0.1 M; this relatively high concentration was chosen to eliminate the effects of small deviations in pH, and to buffer certain strongly ionizing organic osmolytes, particularly taurine. Because the pH optimum studies suggested that "20%" LDH might be a different isozyme from enzymes obtained from osmoconforming worms, this enzyme was studied together with "100%" LDH in many of the subsequent experiments.

*Effects of neutral salts*

Three cations and three anions were combined in all possible ways, at 0.1, 0.25 and 0.5 M, a range which brackets both the normal intracellular ion concentration and that of full strength sea water. Potassium, sodium and chloride were chosen because they are ions prevalent in cells, body fluids or sea water; ammonium, sulfate and acetate, for their predicted lyotropically favorable effects. Assays were run at saturating substrate concentrations. Data are presented for both cationic and anionic series (Figs. 3 and 4) to permit separate analysis of positive and negative ion effects. Each point is an average of two runs, and the bars represent the range.

It is evident that, as the concentration of salt increases, inhibition becomes greater as compared to the activity in a buffered solution alone; both cations and anions play a role in inhibiting the enzyme. Only in the case of ammonium sulfate on "100%" LDH is there an absence of severe inhibition at high (0.5 M) salt concentration. Other ammonium salts have similar effects on both the "100%" and "20%" enzymes. Sodium ions and especially potassium ions are highly deleterious for "100%" LDH, even at low (0.1 M) and intermediate (0.25 M) concentrations, whereas "20%" LDH is less affected by these ions except at high (0.5 M) concentrations. Neither acetate ions nor sulfate ions are capable of significantly offsetting the inhibitory effects of these cations on "100%" LDH.

*Effects of nitrogenous osmolytes*

A number of neutral amino acids, taurine, betaine and TMAO were tested for their effects on "100%" and "20%" LDH activity, at concentrations up to 0.5 M where solubility permitted. One to three replicates were run at each concentration, again using saturating substrate concentrations and 0.1 M TRIS buffer.

The effects of neutral amino acids are shown in Figure 5. As with the salts, the activity of "20%" LDH is, in general, relatively greater at a given osmolyte concentration than is that of "100%" LDH, but the differences are much smaller. With the possible exception of alanine, the aliphatic amino acids are only marginally inhibitory, even at high (0.5 M) concentrations; the longer the chain length, the more innocuous the amino acid, so that leucine and isoleucine have little effect on activity, even at 0.5 M. Likewise, the longer of the two hydroxyamino acids, threonine, is more innocuous than serine. Thus, the more nonpolar an amino acid, the less its inhibitory effect.

The effects of taurine, betaine and TMAO are shown in Figure 6. The tri-

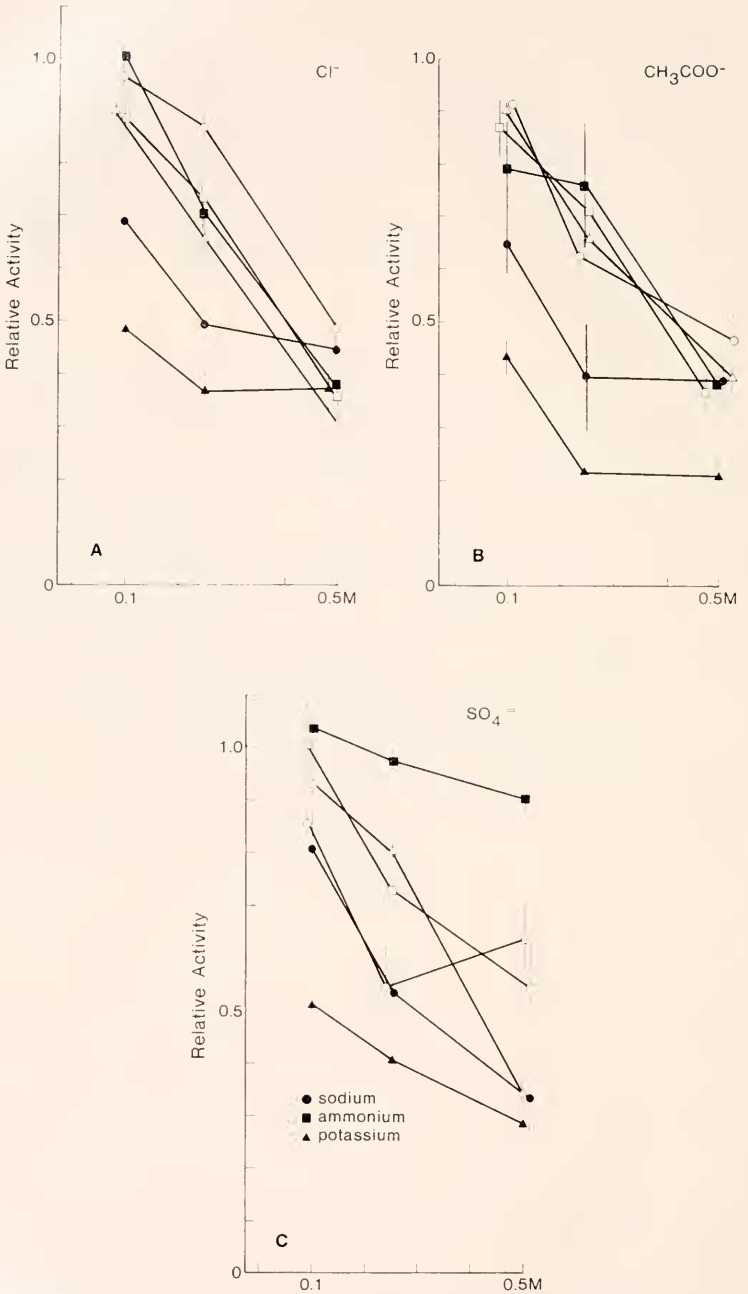


FIGURE 3. Effects of cations on LDH activity with (a) chloride (b) acetate and (c) sulfate counterions. Circles represent sodium; squares, ammonium; and triangles, potassium. Filled symbols are "100%" LDH; open symbols are "20%" LDH. Bars indicate range of two replicates.

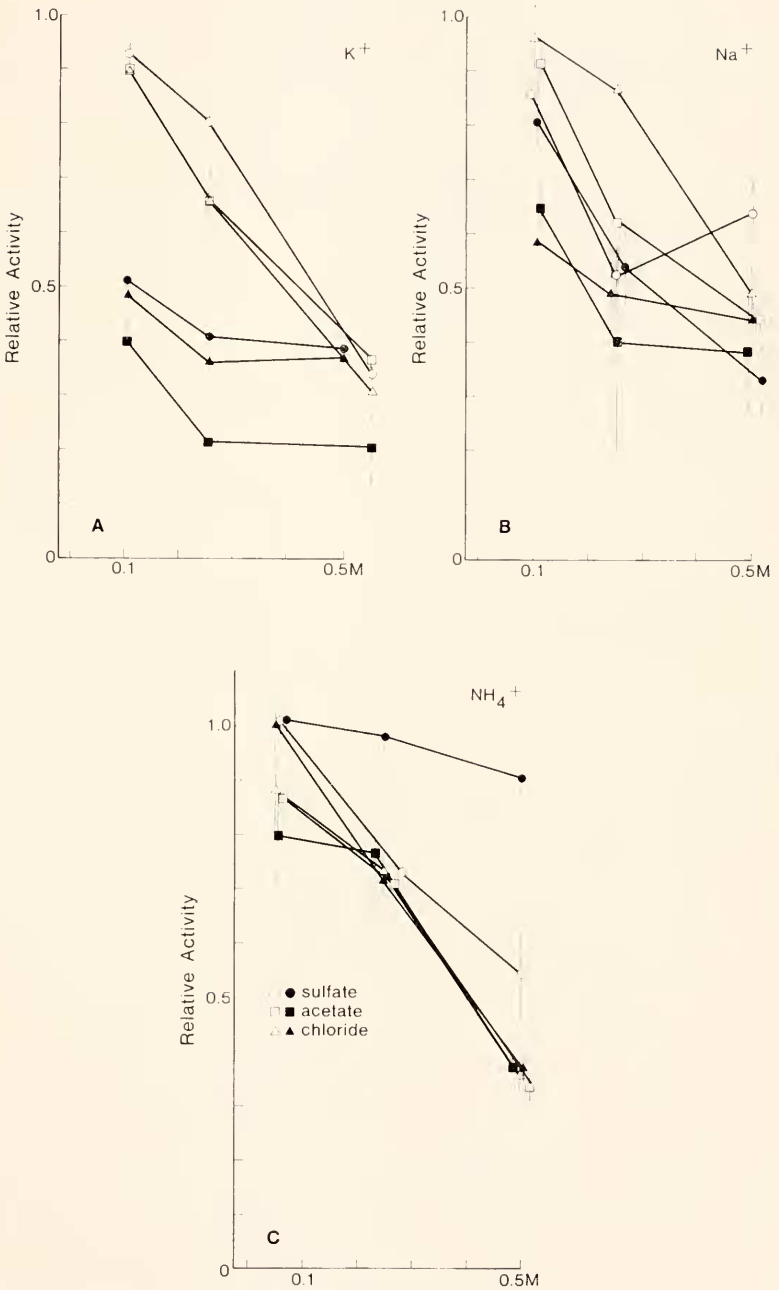


FIGURE 4. Effects of anions on LDH activity with (a) potassium (b) sodium and (c) ammonium counterions. Circles represent sulfate; squares, acetate; and triangles, chloride. Filled symbols are "100%" LDH; open symbols are "20%" LDH. Bars indicate range of two replicates.

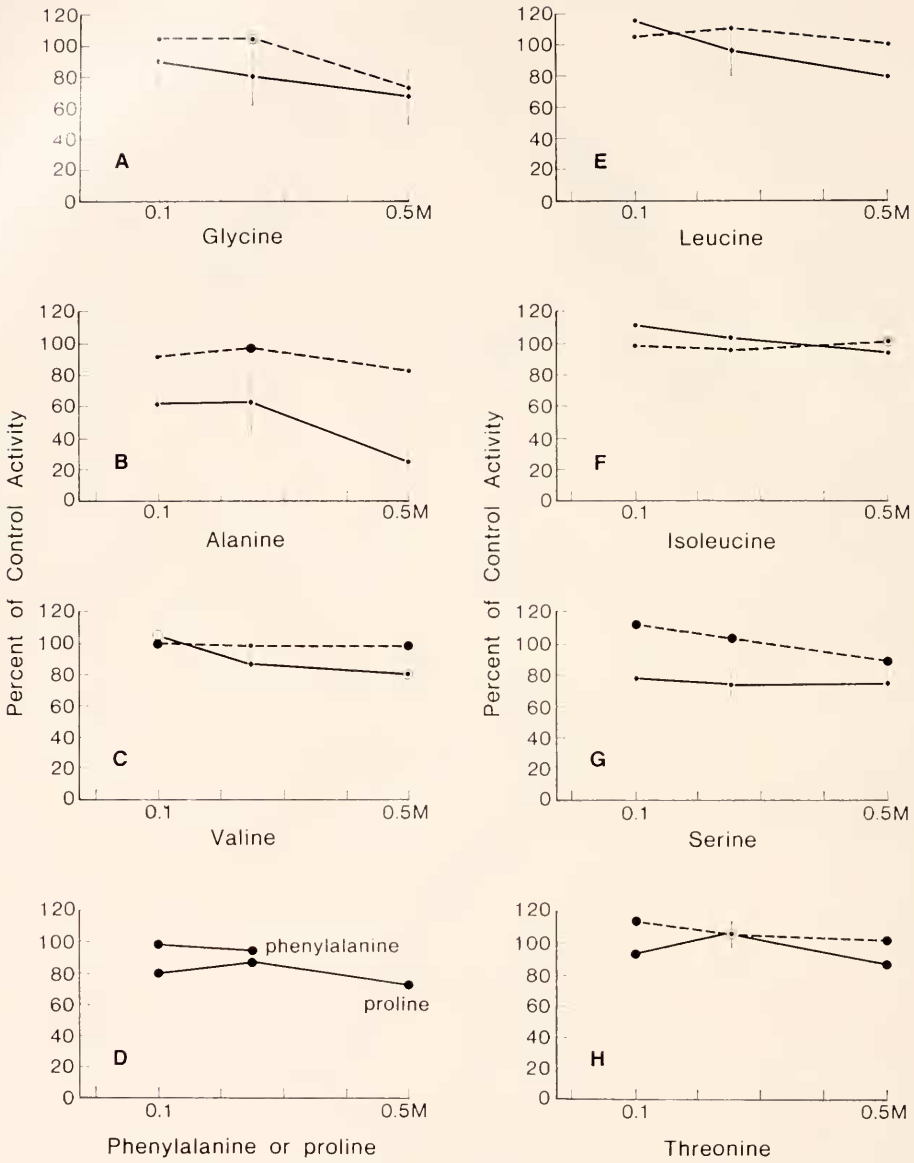


FIGURE 5. Effects of neutral amino acids on LDH activity. Solid lines are "100%" LDH; dashed lines are "20%" LDH. Symbols for replicate agreement are: circle with dot, two replicates differ by >20%; filled circle, two replicates differ by >10%; open circle, two replicates differ by <10%; dot with line, standard deviation of three replicates; dot, single determination.

substituted amines are relatively innocuous; whereas taurine, despite careful buffering, is inhibitory, especially to "100%" LDH.

When TEA or TMA was substituted for TRIS as buffer, there was generally

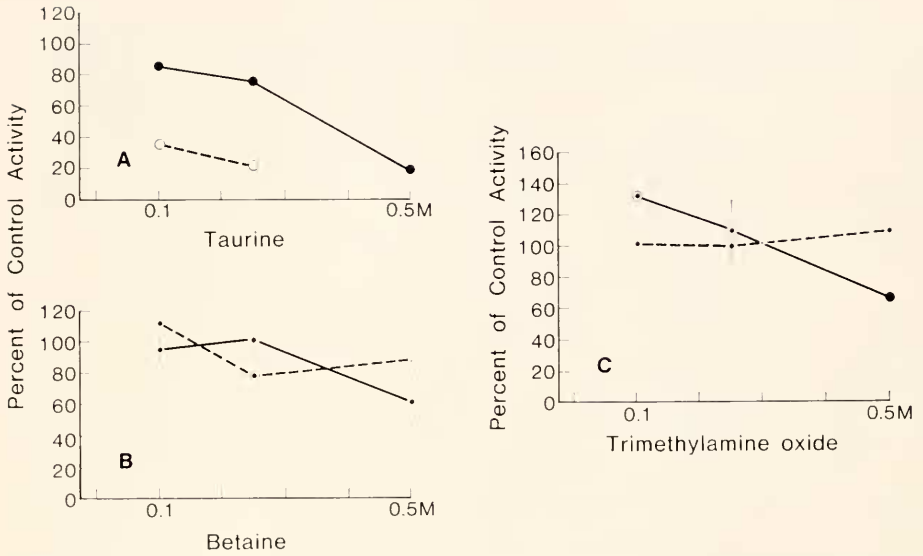


FIGURE 6. Effects of taurine, betaine and TMAO on LDH activity. Solid lines are "100%" LDH; dashed lines are "20%" LDH. Symbols for replicate agreement are the same as in Figure 5.

little difference in enzyme activity, either in the controls or in the presence of various nitrogenous organic solutes. An exception occurred in the case of taurine, however. Compared to the 0.1 M TRIS control, 0.25 M taurine plus 0.1 M TRIS gave about 70% activity; substitution of TEA for TRIS gave about 85% activity, and of TMA, about 120%. It is thus apparent that significant synergistic interactions may occur among cell solutes that, with our present knowledge, cannot be predicted from data on the effects of individual solutes alone.

*Effects of solutes on apparent $K_m$ of pyruvate*

In order to evaluate the probable physiological effects of cell solutes on LDH activity, the apparent pyruvate $K_m$ (substrate concentration where $v = V_{max}/2$) in the presence of various added solutes was measured from double reciprocal plots of $1/v$ versus $1/(S)$. The results are shown in Table I, together with values of $r^2$, the coefficient of determination, which expresses the goodness of fit of the regression. Given the error observed above in replicate determinations, we consider that the slopes are linear in all cases; furthermore, $K_m$ values differing by less than a factor of two are not considered to be significantly different.

The results for "100%" and "20%" LDH are not significantly different. Glycine at 0.25 M has no significant effect on $K_m$, whereas KCl at 0.375 M and 0.5 M approximately triples the the pyruvate concentration required for half maximal velocity. The further addition of glycine has little effect, suggesting that intracellular glycine does not reverse the inhibitory effects of neutral salts, but merely acts as an innocuous substitute osmolyte. On the other hand, 5% bovine serum

TABLE I

*Effect of selected solutes on the apparent* $K_m$ *of pyruvate for "100%" and "20%" LDH.*

| Assay conditions | $K_m$ of pyruvate moles $\times 10^{-4}$/liter | $r^2$ value (coefficient of determination) |
|---|---|---|
| "100%" LDH | | |
| 0.1 M TRIS, pH 7.0 buffer only | 3.7 | 0.925 |
| 0.25 M glycine | 6.2 | 0.876 |
| 0.375 M KCl | 17.1 | 0.929 |
| 0.5 M KCl | 15.6 | 0.995 |
| 0.25 M glycine + 0.375 M KCl | 18.0 | 0.860 |
| 5% BSA | 5.8 | 0.955 |
| 0.25 M KCl + 5% BSA | 6.7 | 0.998 |
| "20%" LDH | | |
| 0.1 M TRIS, pH 7.0 buffer only | 4.6 | 0.998 |
| 0.25 M glycine | 5.6 | 0.978 |
| 0.375 M KCl | 13.9 | 0.995 |
| 0.5 M KCl | 19.9 | 0.975 |
| 0.25 M glycine + 0.375 M KCl | 15.1 | 0.933 |

albumin (BSA) is capable of reversing the deleterious effects of 0.25 M KCl on the apparent pyruvate affinity, although alone its does not enhance substrate binding.

## DISCUSSION

The main emphasis of this study has been on the effect of various intracellular solutes on the activity of LDH obtained from *Nereis succinea*. Before discussing these results, however, it is necessary to consider whether we are dealing with a single enzyme or several isozymes. Long and Kaplan (1968) found that LDH from the related polychaete, *Nereis virens*, is specific for D-lactate, in contrast to the L-specific LDH's of mammalian tissues (Kaplan, 1964). D-LDH's exist as dimers, in contrast to the tetrameric L-LDH's of mammals, and typically have molecular weights of 65,000 to 75,000 daltons (Long and Kaplan, 1973). It is thus likely that a dimeric D-LDH also occurs in *Nereis succinea*. This would permit the existence of three forms of LDH, assuming the presence of two different subunits and, indeed, three LDH bands can be detected after gel electrophoresis of *N. succinea* homogenates (Jones, 1970; Gordon Lusk, San Diego State University, unpublished data). Whether these isozymes have different catalytic properties, however, remains to be determined. Simple hyperbolic behavior with a given homogenate was observed in the present experiments, suggesting that if several isozymes are present, they are kinetically similar.

On the other hand, the data on the differential effects of osmolytes on "100%" and "20%" LDH's suggest that osmotically adaptive isozymes may be produced by *Nereis*, despite the fact that Lusk found no detectable differences in LDH isozyme patterns in homogenates from 100% and 20% SW-adapted worms. At least one species, the brook trout, is known to be capable of forming environmentally adaptive LDH isozymes, in this instance in response to acclimation to various temperatures (Hochachka and Lewis, 1971). Even though evidence for the

presence of osmotically modulated LDH isozymes is not conclusive, it is interesting to speculate briefly on the possible adaptive significance of a low tissue concentration of a salt-sensitive LDH in osmoconforming worms ("100%" LDH) and a doubled tissue concentration of a salt-resistant enzyme in osmoregulating worms ("20%" LDH). Since an alternative pathway for pyruvate is reductive amination to alanine, at high osmolarity, when a large amino acid pool is maintained, low tissue levels of a potassium ion-inhibited LDH would favor alanine synthesis. Conversely, during osmoregulation when the amino acid pool is lowered (while potassium ion concentration remains little changed), an increase in tissue LDH, of a form no longer sensitive to ion inhibition, would facilitate accumulation of lactate.

Although differences in solute effects on "100%" and "20%" LDH's from *N. succinea* appear to exist, the overall responses of the enzymes to various categories of solutes are sufficiently similar that they can be considered together in the subsequent discussion. In general, neutral salts at concentrations equivalent to those found in sea water or extracellular fluids of *Nereis succinea* (Freel *et al.*, 1973) are highly inhibitory for LDH activity and tend to follow the lyotropic series. On the other hand, with the exception of taurine and possibly alanine, nitrogenous solutes appear to be largely innocuous for LDH activity. The fact that acetate ion, alanine and taurine are all far more inhibitory than predicted by our initial hypothesis may be explained by the similarity of these osmolytes to the substrate, pyruvate. Marginal competitive inhibition may be occurring, but this has not been tested. The possibility of species specificity in the activation or deactivation of LDH by various solutes must also be considered. Taurine, for example, although present in most marine invertebrates, ranges widely, from 6 to 106 μmoles per gram wet muscle tissue, even among species of a single phylum, the molluscs (Awapara, 1961; Schoffeniels and Gilles, 1972). Co-evolution of macromolecular structure with solute composition remains as a possible explanation in this instance.

The mechanism by which certain neutral salts partially inhibit enzyme activity is not yet completely clear, although the work of Somero *et al.* (1977) strongly suggests that it is not the catalytic site *per se*, but rather the catalytic conformational changes of the enzyme that are being affected. The effective salt concentrations seem too high for the ions to be acting as specific active site or allosteric site inhibitors; $K_i$ would be in the range 0.1 to 1.0 M, depending on the salt. Thus, although complete denaturation has obviously not occurred, 0.5 M concentrations of salts such as NaCl and KCl must be modifying the conformational structure of the enzyme sufficiently to affect its rate limiting steps. The present results indicate that not only is the overall catalytic rate of the enzyme greatly affected by neutral salts, but that the apparent substrate binding affinity is also significantly reduced (Table I). Hence, an enzyme from a euryhaline polychaete whose body fluids contain high salt concentrations (Freel *et al.*, 1973) is similar to mammalian enzymes (Warren *et al.*, 1966; Warren and Cheatum, 1966) in its susceptibility to salt inactivation.

In the case of LDH, one possible effect of such salts is to cause dissociation of subunits, as demonstrated by Eichner (1973) on LDH from the lobster, *Homarus americanus*. He observed that ammonium sulfate raised to a concentration of 1.3 M caused dissociation of tetramers to dimers, with concomitant loss of activity. However, since such charge-initiated subunit dissociation is unique to this species, and

requires a very high ionic strength, it is unlikely to account for the inhibitory effects of uni-univalent salts at 0.2 to 0.5 M, nor can it explain the fact that different salts have different effects at similar ionic strengths. Other, more subtle salt-induced changes in conformation appear to be affecting catalytic activity. One theory, suggested by Klotz (1965) and modified and elaborated by Lewin (1974) is that neutral salts differ in their action at the macromolecule-solvent interface, thus affecting the quantity and quality of the hydration sphere which in turn determines macromolecular conformation.

The results obtained here on a representative macromolecule, LDH, thus tend to confirm our first two hypotheses: first, that macromolecules of a marine invertebrate are indeed susceptible to functional inhibition by concentrations of those neutral salts found in its environment; and secondly, that lyotropically more favorable salts, such as ammonium sulfate, or nitrogen-containing organic solutes with comparable functional groups, such as amino acids or TMAO, are innocuous to macromolecular function. Although not all nitrogenous solutes are equally favorable toward LDH function, in general they are considerably less deleterious than the major intracellular ions, potassium, sodium and chloride. Since, according to this hypothesis, it is an osmotic equivalence rather than a molar equivalence of neutral salts that must be replaced by organic solutes, the results on several solutes were compared by plotting them as a function of both molarity and osmolarity (Fig. 7). The osmotic equivalence scale is based on the assumption of equal osmotic coefficients for all solutes. This gives a conservative comparison between salts and amino acids, since 0.5 M KCl, for example, has an osmotic coefficient of about 0.90 (Scatchard, Hamer and Wood, 1938) whereas that of 0.5 M amino acids is around 1.0 (Robertson, 1975).

So far, however, there is no proof that the third hypothesis is correct, namely that small nitrogenous organic solutes can offset the effects of functionally deleterious solutes. In the single attempt to test this, the amino acid glycine was without significant effect on the apparent $K_m$ of pyruvate, either in the presence or absence of salts. Results with this one amino acid, however, may not reflect the potential of less polar amino acids, such as valine and isoleucine, to protect the enzyme in the presence of neutral salts. Nor do other researchers appear to have critically tested whether or not solutes capable of stabilizing macromolecular conformation, such as amino acids and ammonium sulfate, are also able to protect them against denaturation by lyotropically active ions.

Of special interest in this regard is the observation that 5% BSA is capable of suppressing the increase in apparent $K_m$ of pyruvate induced by moderate KCl concentrations (Table I). Although intracellular chloride ion concentrations in *Nereis succinea* are low (33 mM in 100% SW-adapted worms) the potassium ion concentration is about 234 mM (Freel *et al.*, 1973); and it is this ion which is most deleterious to "100%" LDH, 0.25 M $K^+$ resulting in 60 to 80% reduction in activity regardless of the counterion present (Figure 3). Although the intracellular soluble protein levels in *Nereis* have not been measured, recent studies on the total intracellular dry weight components of muscle from another marine invertebrate, the giant barnacle *Balanus nubilus,* indicate that soluble macromolecules capable of separation by ultracentrifugation are present at a concentration of some 7 g per 100 g cell water. More than half of this material is protein (M. Clark, unpublished observations). If comparable amounts of soluble intracellular protein are present

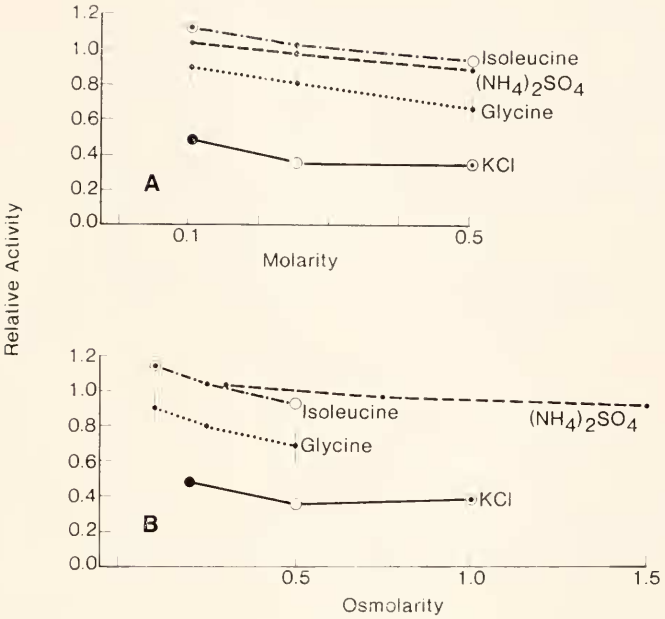


FIGURE 7. Effects of various solutes on the relative activity of "100%" LDH expressed in terms of (a) molarity and (b) osmolarity. Symbols for replicate agreement are the same as in Figure 5.

in *Nereis*, then a 5% BSA solution is a reasonable approximation to the normal intracellular state.

These experiments suggest that small nitrogenous solutes act as relatively innocuous substitute osmolytes for deleterious ions when water is limiting in a cell and that the soluble cytoplasmic proteins play a role in protecting other macromolecules against the functionally disruptive effects of the ions that remain. It is also possible that less polar nitrogenous osmolytes may act to offset the deleterious effects of ions or more polar organic solutes, as suggested both by the fact that less polar amino acids are more innocuous to enzyme function, and by the remarkable improvement in LDH activity observed on substituting less polar TMA for more polar TRIS in the presence of the highly polar solute, taurine. Nonpolar side groups may act at the macromolecular-water interface to increase water activity and, hence, decrease bound water at the surface. There would therefore be a decrease in activation energy for conformational change during catalysis (Lewin, 1974).

Given the fact that even 0.1 M $K^+$ reduced "100%" LDH activity by 50% (although its effect on "20%" LDH is much less), one may consider whether intracellular potassium ion in freshwater or terrestrial species may not also be potentially deleterious to the function of their macromolecules. For example, the intracellular potassium ion concentration in frog (*Rana pipiens*) muscle is 141 mM (Lee and Armstrong, 1974), and in various rat muscles it ranges from 142 to 178 mM (Drahota, 1961). Either the enzymes and other macromolecules of these

species are, like "20%" LDH from *Nereis*, relatively unaffected by such potassium ion concentrations, as appears to be the case for halibut muscle LDH (Somero *et al.*, 1977), or they are protected by soluble protein or other solutes within the cell, or they are functioning at sub-optimal levels. The general applicability of the results observed here, both in terms of other species and other macromolecules, remains to be investigated. These experiments indicate the potential importance of the entire complement of intracellular solutes in determining the physiological level of activity of cell macromolecules.

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## Summary

1. In searching for an explanation for the high intracellular concentrations of nitrogenous organic osmolytes found in all marine invertebrates, the similarity of their functional groups to those neutral salts known to stabilize native macromolecular conformations was noted. This led to the hypothesis that such osmolytes are innocuous and substitute for more deleterious salts at high osmotic pressures. The hypothesis was tested on a representative macromolecule, the enzyme lactate dehydrogenase (LDH), from the euryhaline polychaete, *Nereis succinea*.

2. The activity of LDH from worms adapted to a range of salinities was measured at several pH values and in the presence of various concentrations of neutral salts and of low molecular weight nitrogenous solutes characteristically found intracellularly in marine and euryhaline invertebrates.

3. Differences in LDH activity in homogenates from worms adapted to osmoconforming (100%–35% SW) and osmoregulating (20% SW) salinities were observed. Enzyme activity from osmoregulating worms ("20%" LDH) occurred at nearly twice the concentration in tissue, and was less inhibited by neutral salts than was "100%" LDH. These differences may reflect the synthesis of a new isozyme during osmoregulatory adaptation, designed to divert pyruvate from entering the free amino acid pool by more readily converting it to lactate.

4. Low molecular weight nitrogenous solutes are generally far less inhibiting to LDH activity than are intracellular ions at similar osmolarities. The more nonpolar the compound added, the more innocuous its effect on the enzyme. This may be due either to suppression of charge-initiated dissociation of subunits, or the nonpolar groups may stabilize the macromolecule-solvent interface.

5. Although glycine is unable to protect LDH from inhibition by neutral salts, 5% bovine serum albumin is effective in this respect. This suggests that soluble cytoplasmic protein and perhaps less polar nitrogenous solutes as well play an important role in protecting macromolecules from functional inactivation by ions normally occurring in the cell.

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